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Identification of a Bacterium Which Produces Substances Having Antifungal Activity against Many Important Phytopathogenic Fungi

Tsuneo NAMAI Kiyotaka HATAKEDA*
and Takashi ASANO*

Laboratory of Plant Pathology, Faculty of Agriculture, Tohoku University, Sendai 980, Japan
**Government Industrial Research Institute, Tohoku, Sendai 983, Japan.*

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Summary

A bacterium, which produces antifungal substances in a medium, was isolated. The bacterium inhibited the growth of many phytopathogenic fungi such as *Pyricularia oryzae*, *Helminthosporium oryzae*, *Rhizoctonia solani* and other fungi.

This bacterium is rod-shape measuring 0.7-0.8×2.0-3.5 μm, Gram positive and forms heat-resistant endospore. This bacterium is able to hydrolyze starch but not hippurate and reduces NO_3^- to NO_2^-. In addition, from morphological and physiological properties, this bacterium was identified as a strain of *Bacillus subtilis*.

Rice blast disease, caused by *Pyricularia oryzae* Cavara, is one of the most important crop diseases in Japan. For control of this disease, many kinds of fungicides have been used. Some of these fungicides are antibiotics such as Kasugamicin and Blastcidin produced by Actinomcetes.

The authors isolated a bacterium contaminated during mono-conidial isolation of the rice blast fungus, *P. oryzae*, from lesions on infected rice leaves. The bacterium inhibited remarkably the hyphal growth of *P. oryzae* and many important phytopathogenic fungi on agar plate. It is an interesting matter whether the bacterium can be utilized in biological control of plant diseases or whether the substance produced by this bacterium can be used as a fungicide. The first step of this experiment was carried out in order to identify the bacterium.

Materials and methods

Antagonism to several important phytopathogenic fungi

In order to study the antagonism of the bacterium against phytopathogenic fungi, 7 isolates of *Pyricularia oryzae* and an isolate of *Helminthosporium oryzae*, *Rhizoctonia solani*, *Botrytis cinerea*, *Alternaria solani*, *Fusarium oxysporum* f. sp. *melonis* and *Leptosphaeria salvinii* were used. Mycelial plugs of each fungus were placed 3 cm apart from the point of inoculum of this bacterium on potato sucrose agar (PSA) plate and cultured for 6 days at 25°C in darkness. Growth inhibition of the test fungi in diual culture was expressed as inhibition percentage of the radial mycelial growth rate by the formula: (radial growth rate in single culture − radial growth rate in diual culture/radial growth rate in the single culture) $\times 100$ (4).

Observation by electron microscope

The bacterium cultured on PSA slant was suspended in distilled water, mounted on sheet mesh, dried under vacuum and shadowed by Pt-panasium. The preparation was then observed with a scanning electron microscope. For transfer electron microscopic observations, PSA blocks containing the bacterium were fixed in 1% osmium tetraoxide in phoshate buffer at 4°C for 4 hr, dehydrated with ethanol and propylenoxide series and embedded in Epon 812. Ultra thin sections were cut on a Porter-Blum ultramicrotome with glass knives. The

TABLE 1. *Antagonistic effect of this bacterium against Pyricularia oryzae and other plant pathogenic fungi*

Fungus	Isolate	Inhibition(%)
Pyricularia oryzae	ken 60-19	87.7
	ken 54-20	90.0
	naga 64-8	87.7
	ken 53-33	87.7
	TH 67-22	90.0
	F 67-54	90.0
	ken 62-89	87.7
Helminthosporium oryzae	Kyoto Univ. 13	53.3
Rhizoctonia solani	B-5	56.5
Botrytis cinerea	I-23-1	73.3
Fusarium oxysporum f. sp. *melonis*	I-31-5	33.3
Alternaria solani	I-24-3	50.0
Leptosphaeria salvinii	MK 1-1	73.3

*Inhibition percentage of radial mycelial growth

sections were stained with saturated ulanyl acetate solution for 30 min and followed with lead citrate for 2 min at room temperature and examined with JEM 100 B electron microscope (Japan Electric Optics Laboratory Co., Ltd.)

Cultural and morphological characteristics

The colonial appearance of this bacterium was observed after culturing on PSA plate at 25°C for 6 days. Cell morphology was ascertained from Gram-

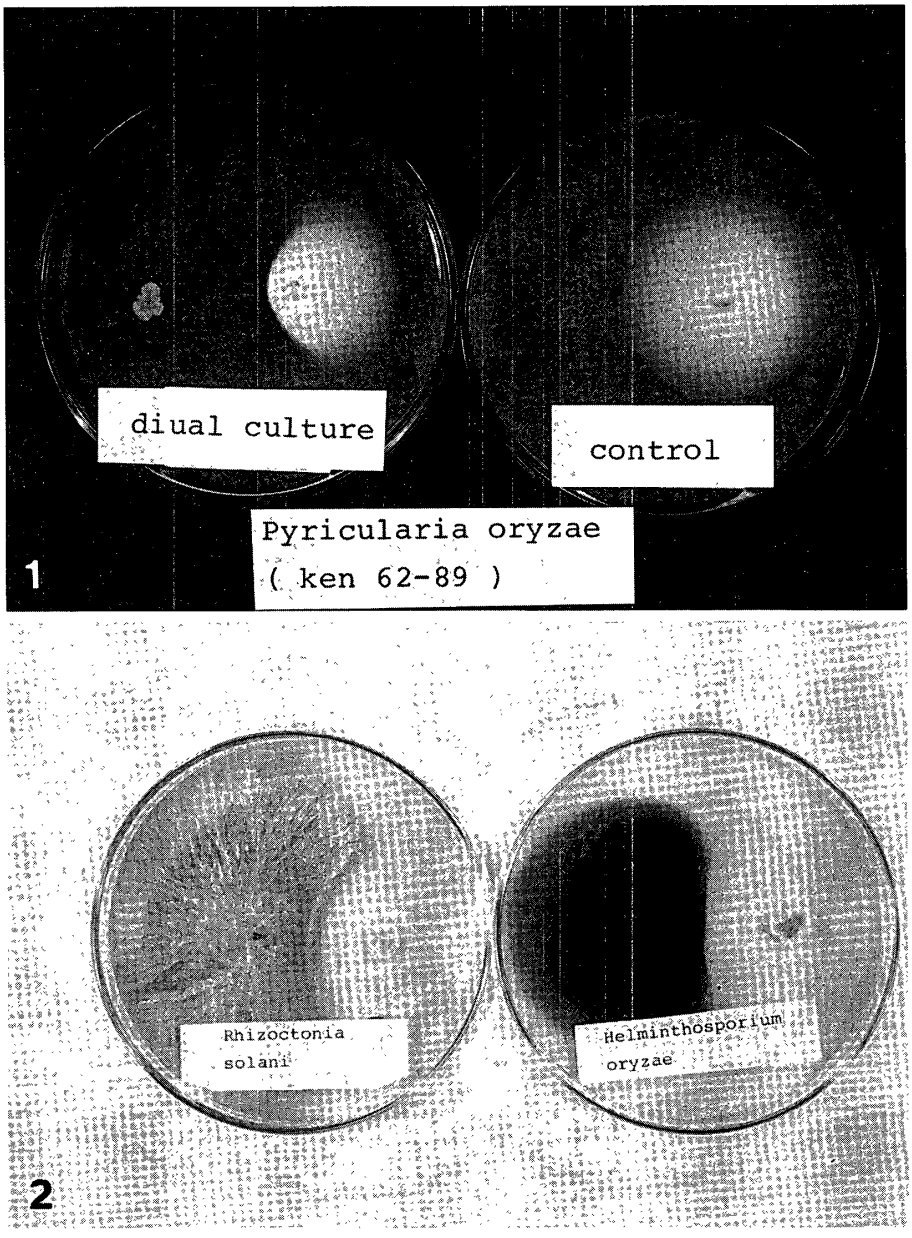

FIG. 1. 2. Antagonism of this bacterium against phytopathogenic fungi.
FIG. 1. Inhibition of hyphal growth to *Pyricularia oryzae*.
FIG. 2. Inhibition of hyphal growth to *Rhizoctonia solani* and *Helminthosporium oryzae*.

staining using the same preparations. Formation of endospore of this bacterium was examined by heat treatment at 80°Cfor 15 min. The reaction to temperature of this bacterial growth on the medium was tested at 5, 9, and 17°C for 2 weeks and at 25, 30, 40, 45 and 50°C for 6 days.

Physiological testing for identification

All testings of properties of this bacterium for identification were tested on the following points: growth in 7% NaCl broth, Sabroued dextrose broth, 0.02% azide broth and anaerobic media; reactions of catalase, litmus milk, egg yolk; casein and gelatin liquerfacation; hydrolysis of starch and hippurate; utilization of citrate and propionate; productions of indol and acid from glucose, arabinose, xylose and mannitol; reduction of nitrate to nitrite; formation of black pigment; Voges-Proskauer test and resistant to lysozyme. These physiological characteristics were tested by the methods described in the references (2, 3, 4, 6).

Results

Antifungal activity against phytopathogenic fungi

Inhibition percentage of hyphal growth of phytopathogenic fungi by diual culture with this bacterium is shown in Table 1. This bacterium inhibited hyphal growth of phytopathogenic fungi used in this experiment. The most sensitive fungus was *Pyricularia oryzae*, and inhibition percentage of hyphal growth of all isolates was ca. 90. Fig. 1 shows the diual culture with an isolate, ken 62-89, *P. oryzae*. Fig. 2 shows the inhibition of hyphal growth of

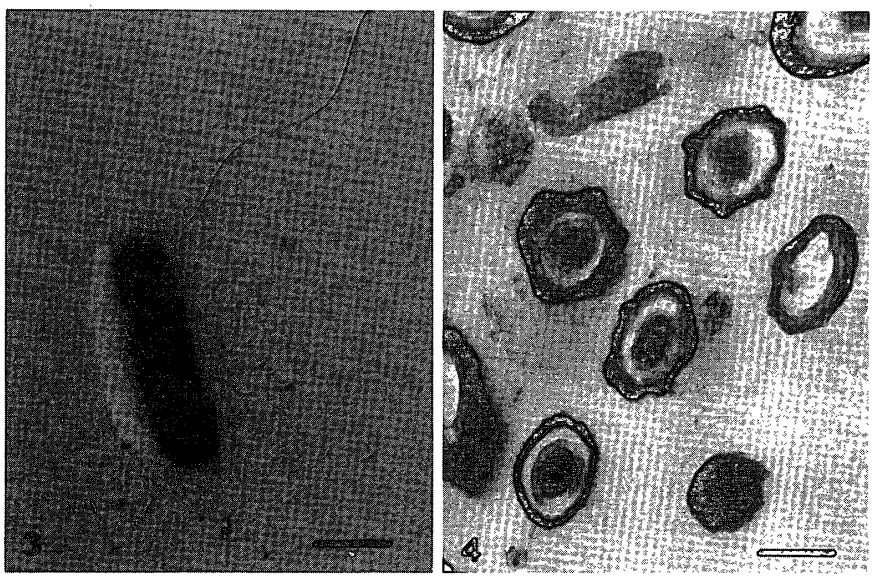

FIG. 3. 4. Electron micrograph of this bacterium
FIG. 3. Bacterial cell with long flagella.
FIG. 4. Endospore formed within a bacterial cell. Scale represented = 1 μm.

TABLE 2. *Physiological characteristics of this bacterium*

Gram staining ;	positive
7% NaCl broth ;	growth
Sabouraud dextrose broth ;	growth
Azide dextrose broth ;	no growth
Formation of black soluble pigment on glucose-nutrient agar ,	negative
on tyrosine-nutrient agar ;	negative
Production of acetyl methyl carbinol ;	positive
Production of acid from glucose ,	positive
from arbinose,	positive
from xylose,	positive
from mannitol ;	positive
Hydrolysis of starch ;	positive
Utilization of citrate ;	positive
Utilization of propionate ;	negative
Resistance to lysozome ;	positive
Production of indol ;	negative
Reduction of nitrate to nitrite ;	positive
Casein ;	decomposition
Litmus milk ;	alkaline degection
Gelatin ;	liquefaction
Tyrosine ;	no decomposition
Hydrolysis of hippurate ;	negative
Catalase ;	positive
Egg yolk reaction ;	negative
Anaerobic growth ;	negative
Growth temperature ;	9 to 50°C

Rhizoctonia solani and *Helminthosporium oryzae* respectively. The inhibition of hyphal growth of these tested phytopathogenic fungi were in the range of 90 to 30%.

Morphological and cultural characteristics

Cells of this bacterium were rod-shaped, 0.7-0.8×2.0-3.5 μm in size and Gram positive. Colonies of this bacterium formed on PSA had irregular margins, wrinkled surfaces and showed creamy colored. When the bacterium was transferred to new PSA medium after treatment at 80°C for 15 min the same characteristic colony was formed. From the investigation and TEM observation, heat-resistant endospores surrounded with cortex were formed inside the cell (Fig. 4). This bacterium was spread actively on an agar plate with a moist surface. Long laterial flagella were found as shown in Fig. 3.

TABLE 3. *Differential characteristics of Bacillus species 1-3 and this bacterium*

	Bacillus subtilis	*Bacillus pumilus*	*Bacillus licheni-formis*	the present bacterium
Rods				
Width, μm	0.7-0.8	0.6-0.7	0.6-0.8	0.7-0.8
Length, μm	2-3	2-3	1.5-3	2-3.5
Hydrolysis of Starch	+	−	+	+
Hippurate	−	+	−	−
NO_3^- to NO_2^-	+	−	+	+
Growth in anaerobic agar	−	−	+	−
Temperature for growth, °C				
Maximum	45-55	45-50	50-55	50
Minimum	5-20	5-15	15	9

Physiological properties

Physiological characteristics of this bacterium are shown in Table 2. The bacterium showed positive reactions in the following tests; catalase, Voges-Proskauer test, growth in 7% NaCl broth, Sabroud dextrose broth, acid production from glucose, arabinose, xylose and mannitol, hydrolysis of starch, utilization of citrate, reduction of nitrate to nitrite, hydrolysis of casein, alkaline degestion in litmus milk, liquefaction of gelatin and resistance to lysozyme.

On the other hand, this bacterium showed negative reaction to following tests; anaerobic growth, egg yolk reaction, utilization of propionate, decomposition of tyrosine, growth in 0.02% azide broth, formation of black pigment, production of indol and hydrolysis of hippurate. The colony of this bacterium was formed on PSA plate in the range of 9 to 50°C.

Discussion

This paper dealt with the identification of a bacterium which contaminated during the mono-conidial isolation of rice blast fungus, *Pyricularia oryzae*, from lesions formed on infected rice leaves and inhibited the hyphal growth of this fungus on PSA plate. From the viewpoints of morphological, cultural and physiological properties referred by Bergey's Manual of Determinative Bacteriology (8 th ed.) and other references (1,6), the present bacterium was identified as a strain of *Bacillus subtilis*.

Henmi mentioned previously that the bacterium isolated from rice leaves inhibited the germination of conidia of *P. oryzae* and *H. oryzae* (5). Sakamoto *et al.* reported that a strain of *Bacillus subtilis* produced antibacterial substance

against phytopathogenic bacteria such as *Xanthomonas campestris* pv. *oryzae*, *Pseudomonas solanacearum* and *Agrobacterium tumefaciens*, but the chemical structure of the substance was not determined (7). It is well known that *Bacillus subtilis* produces many kinds of antibiotic substances (8, 9, 10), but the substance produced by the present bacterium differed from those compounds in molecular weight, composition of amino acid and other chemical properties (unpublished data). Futher work is in progress on determination of chemical structure of this compound.

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